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ORIGINAL ARTICLE

Methotrexate and low-dose prednisolone downregulate osteoclast function by decreasing receptor activator of nuclear factor- κ B expression in monocytes from patients with early rheumatoid arthritis

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ABSTRACT

Objective Rheumatoid arthritis (RA) is a systemic, immune-mediated inflammatory disease that ultimately leads to bone erosions and joint destruction. Methotrexate (MTX) slows bone damage but the mechanism by which it acts is still unknown. In this study, we aimed to assess the effect of MTX and low-dose prednisolone (PDN) on circulating osteoclast (OC) precursors and OC differentiation in patients with RA.

Methods Patients with RA before and at least 6 months after MTX therapy were analysed and compared with healthy donors. A blood sample was collected in order to assess receptor activator of NF- κ B (RANK) ligand surface expression on circulating leucocytes and frequency and phenotype of monocyte subpopulations. Quantification of serum levels of bone turnover markers and cytokines and OC differentiation assays were performed.

Results Classical activation markers of monocytes and RANK increased in patients with RA at baseline, compared with control healthy donors, and after MTX and low-dose PDN (MTX+PDN) exposure they decreased to control levels. Although the number of OC was not different between groups, the percentage of resorbed area and the resorbed area per pit reduced after treatment. Serum soluble receptor activator of nuclear factor-kappa (RANKL) levels increased at baseline compared with healthy donors and normalised after therapy.

Conclusion Our results suggest that MTX+PDN play an important role in downregulating OC function, which we believe occurs through the decrease in RANK surface expression in monocytes.

Rheumatoid arthritis (RA) is a chronic, immune-mediated, inflammatory disease that mainly impacts joints. It affects around 1% of the world population and the majority of the affected patients are women.¹ The main

Key messages

What is already known about this subject?

- Methotrexate slows bone damage but the mechanism by which it acts is still unknown.

What does this study add?

- Methotrexate and low-dose corticosteroids downregulate osteoclast function through the decrease of receptor activator of NF- κ B surface expression in monocytes.

How might this impact on clinical practice?

- These observations reinforce the relevance of early treatment intervention in early RA with low-dose prednisolone and methotrexate.

features of RA are synovial hyperplasia with pannus formation and bone erosions. On the other hand, systemic inflammation leads also to secondary osteoporosis and bone fragility.² In RA, there is an imbalance between bone formation and bone resorption. In fact, the immune system contributes directly to bone resorption by producing receptor activator of NF- κ B (RANK) ligand and indirectly through the secretion of pro-inflammatory cytokines that act synergistically with the RANK-RANKL system, further enhancing osteoclast (OC) differentiation and bone resorption.³ Monocytes are OC precursors; these cells have phenotypical and functional heterogeneity and have a critical regulatory role in inflammation and innate immune response.^{4 5} Monocytes can be subdivided into three subpopulations based on their

expression of CD14 and CD16 surface markers: the classical (CD14^{bright}CD16⁻), intermediate (CD14^{bright}CD16⁺) and non-classical (CD14^{dim}CD16⁺) subsets.⁴ All of these precursors are able to differentiate into OC increasing bone resorption.^{6,7} Moreover, in RA, CD14⁺ monocytes continuously migrate into the injured joint tissues where they are activated and act as local and systemic amplifiers of the disease through a multitude of mediators.⁸⁻¹⁰

Methotrexate (MTX) combined with low-dose prednisolone (PDN) is the cornerstone of early RA treatment, reducing inflammation and slowing down bone damage.¹¹⁻¹⁴ A recent report depicted that OC formation and OC gene expression, such as NFATc1 and DC-STAMP, which are induced by the cytokine RANKL, are significantly inhibited by MTX.¹⁵ In this study, we aimed to assess the effect of MTX and low-dose PDN (MTX+PDN) on the circulating OC precursors and OC differentiation in patients with RA.

PATIENTS AND METHODS

Patients

Patients with early untreated RA (<12 months of disease duration) fulfilling the American College of Rheumatology/European League Against Rheumatism criteria, 2010¹⁶ were recruited from the Rheumatology and Bone Metabolic Disease Department, Hospital de Santa Maria, Lisbon Academic Medical Centre, Portugal. All patients included were naïve to disease-modifying anti-rheumatic drugs (DMARDs) and corticoids and were followed in the outpatient clinic during a minimum of 6 months after starting MTX+PDN treatment. Moderate-to-high disease activity was required for inclusion, defined as disease activity score 28 (DAS28) >3.2.¹⁷ Patients previously exposed or actively under corticoids and/or DMARD therapy were excluded from this study. The use of non-steroidal anti-inflammatory drugs was not an exclusion criterion. Information regarding patients' demographics, duration of symptoms, erythrocyte sedimentation rate (ESR), C reactive protein (CRP), tender and swollen joints counts, presence of erosions, presence of rheumatoid factor (RF) and presence of anticitrullinated protein antibodies was collected. Both DAS28 and the Health Assessment Questionnaire¹⁸ were applied to patients. Patients with RA were managed by the standard-of-care approach, with no deviation induced by the participation in this study.

Heparinised blood and serum were collected from each participant at the starting of MTX and prednisolone and after 6 months of reaching the minimum effective dose of MTX. Blood was used for flow cytometry and peripheral blood mononuclear cell (PBMC) isolation. Healthy donors matched for age and gender were used as controls. The local ethics committee approved this study and all participants signed an informed consent. Patients were managed in accordance with the standard practice and the study was conducted in accordance with the Declaration of Helsinki as amended in Brazil (2013).

Antibodies and flow cytometry

Identification of B and T cells and neutrophils in peripheral blood and immunophenotyping of monocytes in the PBMC samples were performed using matched combinations of antihuman murine monoclonal antibodies. For peripheral blood staining, anti-CD19 PerCP-Cy5.5 (eBioscience, San Diego, California, USA), anti-CD3 PerCP (BD Biosciences, Franklin Lakes, New Jersey, USA), anti-CD66b FITC (Immunotools, Friesoythe, Germany) and anti-RANKL PE (Santa Cruz Biotechnology, Dallas, Texas, USA) were used. Monocyte subpopulations were identified with anti-CD14 FITC (BD Biosciences) or PerCPCy5.5 (Immunotools) and anti-CD16 APC (Immunotools) and stained with combinations of anti-CD11b PE-Cy7, CD105 PE, CD62L PE-Cy7, CD51/CD61 FITC (eBioscience), CCR2 PE (R&D Systems, Minneapolis, Minnesota, USA), human leucocyte antigen - antigen D related (HLA)-DR PerCP (BD Biosciences) and RANK PE (Santa Cruz Biotechnology). Cell death was assessed by staining with Annexin V Apoptosis Detection Kit APC (eBioscience). Acquisition was performed using a FACSCalibur (BD Biosciences).

Heparinised whole blood was used for staining. Erythrocytes were lysed with red blood cell lysis buffer and cells were incubated with IgG block solution 300 ng/mL (ChromPure Mouse IgG whole molecule, Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) before staining. Absolute cell counts were calculated from differential leucocyte count determined for all participants. PBMCs were isolated by density gradient centrifugation with Histopaque-1077 (Sigma-Aldrich, St. Louis, Missouri, USA). Monocyte subpopulations were identified as described before based on their CD14 and CD16 surface expression.⁴ Data were analysed using FlowJo software (TreeStar, Stanford University, Stanford, California, USA).

Osteoclast differentiation

PBMCs isolated by density gradient centrifugation were plated in 96-well culture plates as described before.¹⁹ PBMCs were left overnight for osteoclast precursors (OCPs) to adhere on bone slices (Immunodiagnostic Systems, Boldon, UK).²⁰ On the following day (day 1 of culture), medium was changed to Dulbecco's modified Eagle's Medium (DMEM) supplemented with macrophage colony stimulating factors (M-CSF) 25 ng/mL (Peprotech, London, UK). Three days later, medium was again changed to DMEM with M-CSF (25 ng/mL), soluble RANKL (sRANKL) (50 ng/mL; Peprotech), dexamethasone (10 nM; Sigma-Aldrich) and transforming growth factor β (2.5 ng/mL; R&D Systems) in order to differentiate the OCPs into mature OC.²¹ The culture medium was then changed twice a week. Cells cultured on bone slices for 7, 14 and 21 days^{20,22} were used for functional assays and gene expression.

Functional assays

TRAP staining of OC was performed using the Acid Phosphate, Leukocyte Kit (tartrate resistant acid phosphatase (TRAP), Sigma-Aldrich) according to the manufacturer's instructions. This assay allows the identification of mature OC (TRAP-positive cells containing three or more nuclei). In the resorption assay, to measure the resorbed area, OC were removed from the bone slices using sodium hypochlorite and stained with 0.1% toluidine blue.

Bone slices from both TRAP staining and resorption functional assays were photographed in an area of 1.25 mm² with a brightfield microscope (Leica DM2500, Leica, Wetzlar, Germany) under a 10x objective. The number of TRAP-stained OC was counted for each time-point per condition and the resorption pits were traced using ImageJ (NIH, Bethesda, Maryland, USA), a mean value of the resorbed area was calculated and expressed in percentage of total area.

Bone turnover markers detection

Serum sRANKL and osteoprotegerin (OPG) were quantified by ELISA (Biomedica Grouppe, Vienna, Austria) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed with SPSS Statistics V.17.0 (IBM, Portsmouth, UK). Categorical variables were expressed as frequencies and comparisons were

tested using χ^2 test. Continuous variables were expressed by median and IQR. Before and after treatment values within each sample were compared using Wilcoxon's matched-pairs signed-rank test. To compare patients with RA with healthy age-matched and sex-matched donors, Mann-Whitney U test was used. Clinical variables such as DAS28, CRP, ESR and tender and joint count were correlated with OC activation parameters (pit number and carboxy-terminal telopeptide of type I collagen (CTX-I)) using Spearman's correlation; p values <0.05 were considered to be statistically significant.

RESULTS

Population characteristics

This study was conducted in 14 untreated patients with early RA, before and after initiation of combined MTX and PDN treatment, and in 18 age-matched and gender-matched healthy donors. All patients had <12 months disease duration and were corticosteroid and synthetic DMARD naïve at baseline. The clinical and demographic features of patients before and after treatment and of the healthy donors are described in table 1. All patients were under MTX (mean dose of 15 mg/week) and prednisolone (<10 mg/day). To three patients hydroxychloroquine (400 mg/day) was added later on and two patients received concomitant sulfasalazine (2 g/day), due to incomplete response to MTX. Patients' median

Table 1 Summary of patients and healthy controls' characteristics

	Patients with RA h		Healthy	p Value
	Baseline	Treated		
Sample size	14		18	
Age (years)	50 (32–60)		44 (36–52)	0.50853
Females (n, %)	10, 71%		11, 61%	0.7120
Symptoms duration (months)	9 (2–12)		NA	
RF (n positive, % positive)	8, 57%		NA	
ACPA (n positive, % positive)	5, 36%		NA	
Erosive (n positive, % yes)	3, 21%		NA	
Treatment with NSAID (n, %)	8, 57%		NA	
NSAIDs duration (months)	2.3 (0.3–5.5)		NA	
Treatment with MTX (%)	0% 100%*			
ESR (mm/hour)	22 (11–44)	13 (9–27)	NA	0.1934
CRP (mg/dL)	0.3 (0.1–2.7)	0.2 (0.1–0.3)	NA	0.1289
Tender joint count	4 (0–7)	0 (0–1)	NA	0.3738
Swollen joint count	6 (2–10)	1 (0–1)	NA	0.0380*
DAS28	4.7 (4.2–5.4)	2.4 (2.1–3.6)	NA	0.0137*
HAQ	1.4 (0.6–2.2)	0.3 (0.0–0.7)	NA	0.1250

*All patients were under MTX and prednisolone. To three patients hydroxychloroquine was added later on and two patients received concomitant sulfasalazine. *p<0.05 between patients before and after treatment with MTX.

Data are represented as median and IQR unless stated otherwise.

RA, rheumatoid arthritis; NA, not applicable; RF, rheumatoid factor; ACPA, anticitrullinated protein antibodies; NSAIDs, non-steroidal anti-inflammatory drugs; MTX, methotrexate; ESR, erythrocyte sedimentation rate; CRP, C reactive protein; DAS, disease activity score; HAQ, Health Assessment Questionnaire.

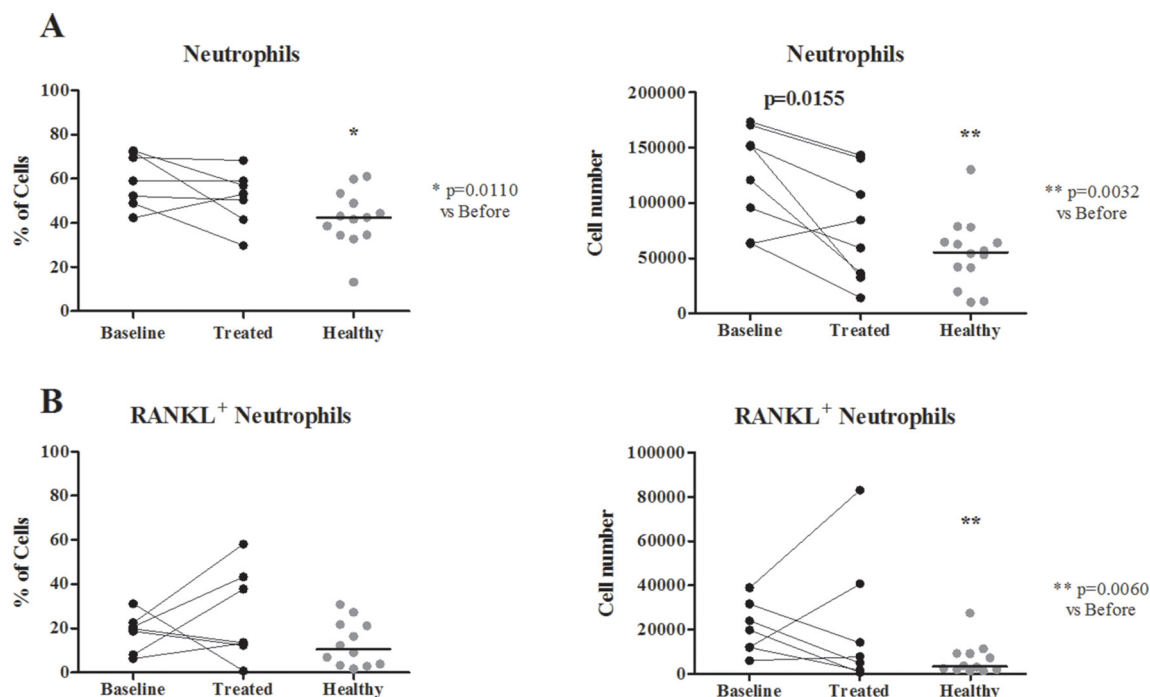


Figure 1 Frequency of neutrophils and RANKL neutrophils. (A) Neutrophil frequency is significantly higher in patients with RA at baseline when compared with healthy donors (both frequency $p=0.0110$ and absolute number $p=0.0032$). (B) RANKL+neutrophil count is also higher at baseline when compared with healthy donors ($p=0.0060$). Neutrophil number is decreased after MTX and low-dose prednisolone treatment in patients with RA (absolute number only, $p=0.0155$). Each dot represents a sample and the line in healthy represents median. RA, rheumatoid arthritis; MTX, methotrexate; RANKL, receptor activator of nuclear factor- κ B ligand.

MTX therapy duration was 13 months (minimum 6 and maximum 24 months).

Patients with RA had a higher number of neutrophils expressing RANKL than controls, which regressed to control levels after treatment

The number of neutrophils was significantly higher in patients with RA at baseline when compared with healthy donors (both frequency $p=0.0110$ and absolute number $p=0.0032$; figure 1). Moreover, the absolute number of neutrophils expressing RANKL on their surface was also higher than in controls ($p=0.0060$). After treatment, the absolute counts of neutrophils decreased ($p=0.0155$). No difference was found in the total number of circulating T or B cells before and after therapy or when compared with healthy donors (data not shown). Moreover, no differences were found regarding RANKL surface expression on neutrophils, T and B cells.

Treatment reduced RANK expression in monocyte subpopulations in patients with RA

Regarding the monocyte subpopulations (classical $CD14^{\text{bright}}CD16^{-}$, intermediate $CD14^{\text{bright}}CD16^{+}$ and non-classical $CD14^{\text{dim}}CD16^{+}$), no differences in frequency or cell death among the three subpopulations or between groups were found (data not shown).

In patients with RA at baseline, CCR2 expression was higher both in the classical and intermediate subpopulations ($p=0.0120$ and $p=0.0471$ when compared with controls; figure 2) and decreased after therapy ($p=0.0039$

for classical and $p=0.0237$). HLA-DR and CD86 expression were also higher in the classical subpopulation of patients with RA at baseline ($p=0.0002$ and $p=0.0068$) as compared with controls. However, while HLA-DR expression remained elevated after treatment ($p=0.0006$ compared with controls), CD86 expression decreased ($p=0.0237$). RANK expression was higher than in controls, both in the intermediate and non-classical subpopulations ($p=0.0112$ and $p=0.0475$, respectively), while CD51/CD61 ($\alpha_v\beta_3$ integrin) was higher in the intermediate subpopulation ($p=0.0487$) and CD11b in the non-classical subpopulation ($p=0.0160$) as compared with controls. Treatment with MTX+PDN decreased RANK and CD11b in the intermediate ($p=0.0234$ and $p=0.0125$) and non-classical subpopulations ($p=0.0084$ and $p=0.0132$) and CD11b in the classical subpopulation ($p=0.0091$).

In vitro assays showed that OCs from patients with RA at baseline are more active than the ones from treated patients and controls

The number of OC increased in all groups throughout culture time. No significant differences in OC number/ mm^2 or pit number were found (figure 3). However, the percentage of resorbed area was higher in patients with RA at baseline at culture days 14 and 21 when compared with controls ($p=0.0333$ and $p=0.0436$). Resorbed area per pit was higher at culture day 21 before treatment when compared with controls ($p=0.0249$) and decreased after therapy, reaching statistical significance at day 14 of

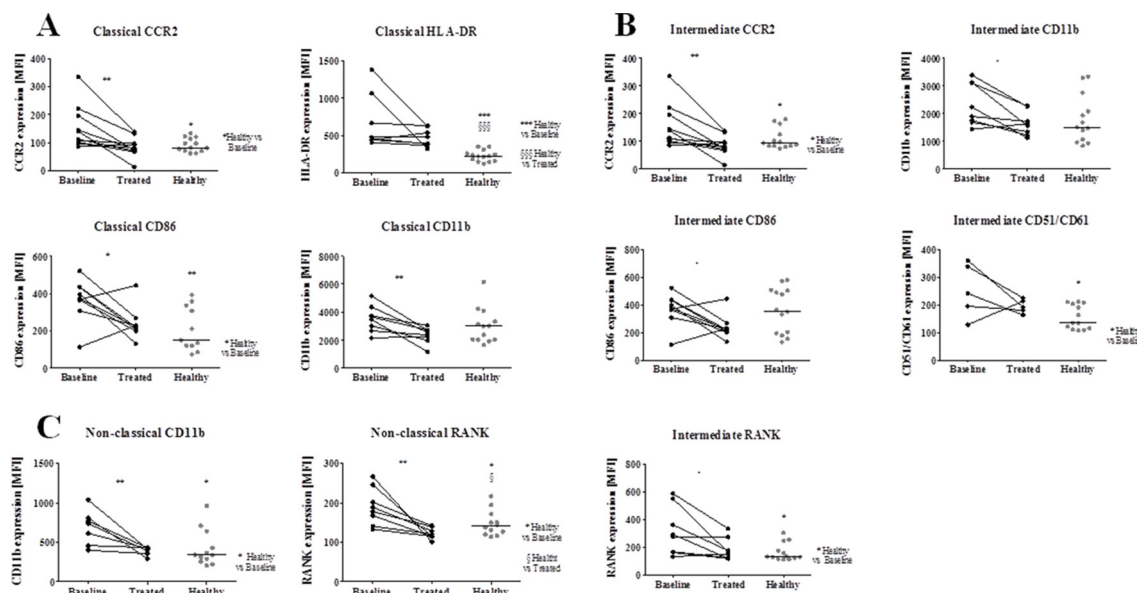


Figure 2 Phenotype of monocyte subpopulations. (A) Classical subpopulation phenotype, (B) intermediate subpopulation, (C) non-classical subpopulation phenotype. Before treatment there was higher expression of CCR2 in classical and intermediate subpopulations, HLA-DR and CD86 in classical subpopulation, CD51/CD61 in the intermediate subpopulation, CD11b in non-classical subpopulation and RANK both in intermediate and non-classical subpopulations. HLA-DR expression remained higher in patients after treatment when compared with controls. CCR2, CD86, CD11b and RANK were decreased in the referred populations after treatment. * $p<0.05$, ** $p<0.01$. Each dot represents a sample and the line in healthy represents median. MFI, median fluorescence intensity; CCR2, C-C chemokine receptor type 2; HLA, human leucocyte antigen; RANK, receptor activator of nuclear factor- κ B; MTX, methotrexate.

culture ($p<0.0001$). Pit number at day 21 was positively correlated with tender joint count when all patients were analysed ($r=0.499$, $p=0.042$).

Bone turnover markers increased in patients with RA at baseline and normalised after treatment

Serum level of sRANKL was higher in patients with RA at baseline than in controls ($p=0.0164$; [figure 4](#)) and normalised after treatment. OPG levels did not change with treatment and was not different from controls. The sRANKL/OPG ratio was higher in patients when compared with controls and it was not affected by treatment ($p=0.0228$ and $p=0.0283$, respectively). The ratio of CTX-I/P1NP positively correlated with DAS28 in patients before therapy ($r=0.786$, $p=0.036$). After treatment with MTX and prednisolone, both CTX-I and P1NP negatively correlated with swollen joint count ($r=-0.913$, $p=0.002$ and $r=-0.756$, $p=0.030$, respectively).

DISCUSSION

In this study, we have shown a decrease in the expression of surface markers on the different monocyte subpopulations, as well as a decrease in OC activity, in patients with early RA treated with MTX and low-dose PDN. The characteristics of these patients were similar to other very early RA cohorts.²³

Neutrophils are involved in RA pathology since the early phase of the disease and they express surface RANKL when activated.^{23–26} We showed that the number of neutrophils and RANKL⁺ neutrophils increased in

untreated patients with RA when compared with controls and that, after treatment with MTX+PDN, the number of neutrophils decreased, reaching control values. Despite also being a source of RANKL,^{27–29} no differences were found in T or B lymphocyte RANKL surface expression before and after therapy.

Patients with RA have increased expression of monocyte surface markers of adhesion, activation and proteins involved in OC differentiation, like RANK and CD51/CD61 ($\alpha_v\beta_3$ integrin).^{30–32} In our study, after exposure to MTX+PDN, CCR2, CD86, CD11b and RANK expression reduced in several monocyte subpopulations, reaching normal expression levels. Dendritic cells, also present in circulation, express CD14, CD16 and CD86^{33–34} and can also differentiate into OC.^{35–36} These cells were also represented in the CD14/CD16 subpopulations of monocytes since we did not exclude them. It was previously shown that CCR2 is upregulated in monocytes and T cells in patients with RA. In vivo studies have shown that this surface marker is involved in recruitment and activation of monocytes in response to monocyte chemoattractant protein-1 (MCP-1).^{37–38} CD86 is a costimulatory molecule involved in the antigen-presenting process and it has been shown to have an important role in T cells activation in RA synovia.³³ CD11b expression is increased in RA monocytes, promoting their binding and migrating properties and it has been shown that corticosteroid therapy decreases CD11b expression.^{39–41} These surface molecules (CCR2, CD86 and CD11b) are important in migration,

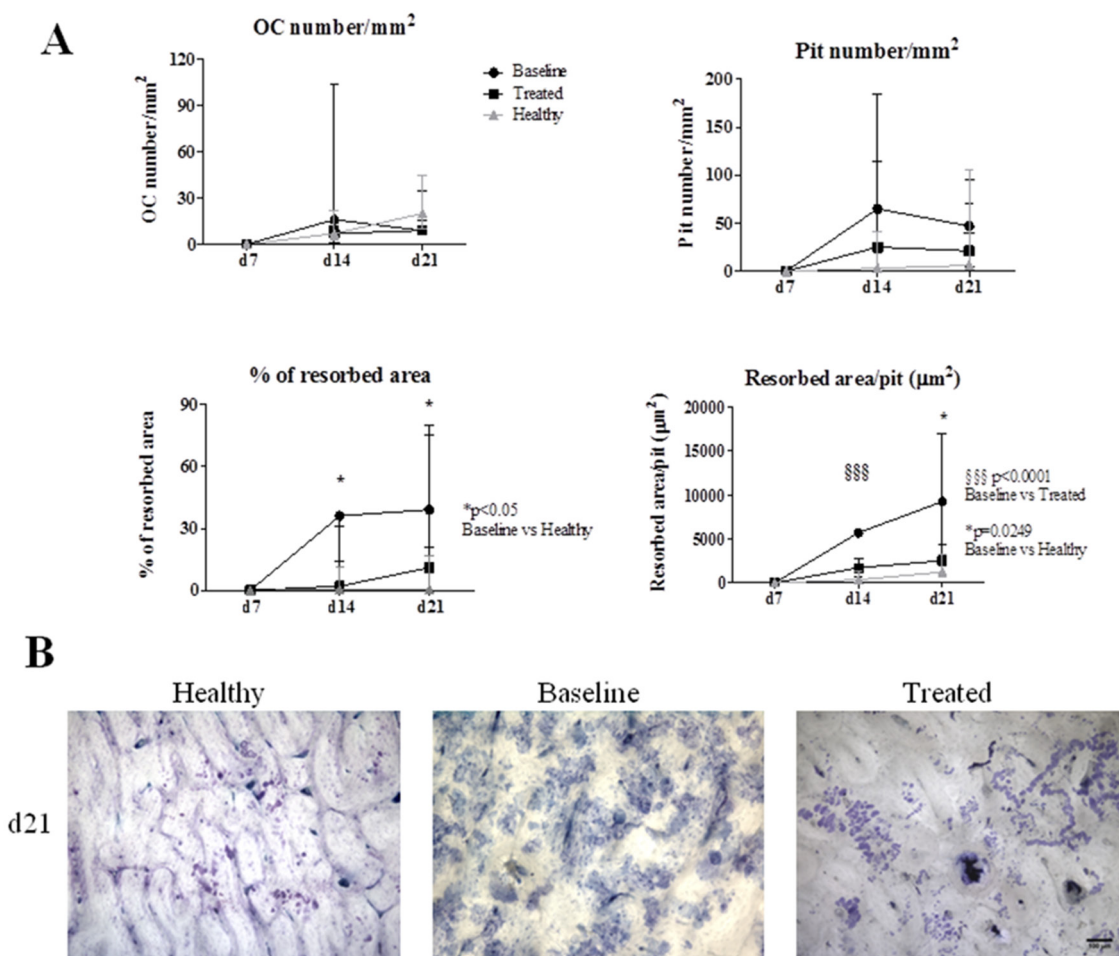


Figure 3 Functional assays in differentiated OC. (A) No differences in OC number/mm² or in pit number were found. Percentage of resorbed area was higher in patients at baseline at culture day 14 and 21 when compared with controls. Resorbed area per pit was lower at culture day 14 when compared with patients at baseline. At culture day 21, patients with RA at baseline had higher resorbed area per pit than controls. Dots and lines represent median and IQR. (B) Representative images of resorption pit assay in all groups at culture day 21; scale bar represents 100 µm. d, day; OC, osteoclast; RA, rheumatoid arthritis.

activation and promotion of inflammation contributing to the continuous inflammatory infiltrate in the synovia. After therapy with MTX and corticoids, these surface markers were reduced in line with a decrease of swollen and tender joints.

We verified that the serum sRANKL/OPG ratio increased in RA, as previously described.⁴² After treatment with MTX and low-dose PDN, the sRANKL/OPG ratio decreased. MTX has been recently associated with both reduction of the RANKL/OPG ratio and of

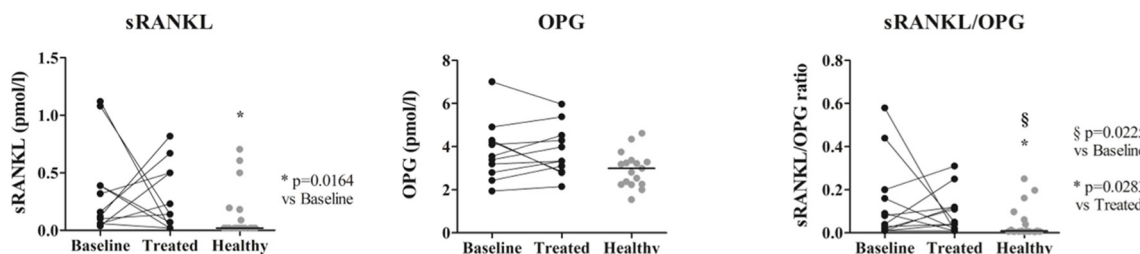


Figure 4 Serum sRANKL and OPG levels in healthy donors and patients with RA at baseline and after MTX and low-dose prednisolone treatment. Both sRANKL and the sRANKL/OPG ratio increased in patients at baseline when compared with healthy donors. There were no differences in circulating OPG level before and after treatment or when compared with controls. The sRANKL/OPG ratio remained significantly higher after treatment. Each dot represents a sample and the line in healthy represents median. sRANKL, soluble receptor activator of nuclear factor-κB ligand; OPG, osteoprotegerin; RA, rheumatoid arthritis.

dickkopf-1 levels⁴³ and it has been previously shown that MTX directly inhibits osteoclastogenesis in vitro in synovial fibroblasts and PBMC cocultures by downregulating RANKL expression on synovial fibroblasts, but no effect on RANK expression was found.^{44 45}

Finally, RANK is the essential receptor for monocyte differentiation in OC, it is upregulated in patients with RA and has been implicated in RA bone loss, both systemic and locally.⁴² We propose that the decrease in monocyte migration/adhesion molecules and downregulation of RANK expression after therapy decrease the osteoclastogenic potential of these cells. In fact, MTX-treated patients with RA reached the maximum OC number later than untreated patients and also than controls and the resorbed area per pit decreased significantly after treatment. This suggests that MTX+PDN have an important role in downregulating OC function, which we believe is at least partially through the decrease in RANK surface expression by monocytes.

In conclusion, our results suggest that the European League Against Rheumatism recommended treatment approach for patients with early RA (MTX combined with low-dose corticosteroids¹⁴) downregulates OC function, which we propose to occur through the decrease in RANK surface expression by monocytes.

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Contributors Conceived and designed the experiments: IPP, JC-L, JEF, HC, MA. Performed the experiments: IPP, JC-L. Analysed the data: IPP, JC-L, CP, RC-M, AMR. Contributed reagents/materials/analysis tools: IPP, RC-M, CP, AMR, HC. Wrote the paper: IPP, JC-L, RC-M, CP, AMR, HC, MA, JEF.

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Competing interests None declared.

Patient consent There is no identifiable individual in this paper. All patients that were enrolled signed the institutional Biobank consent form and it complies with Data Protection Laws from Portugal as approved by our local ethics committee and by the Portuguese National Data Protection Authority.

Ethics approval Ethics committee from Hospital de Santa Maria, Lisbon Academic Medical Centre, Lisboa, Portugal.

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